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Iron Starvation Leads to Oxidative Stress in *Anabaena* sp. Strain PCC 7120

Amel Latifi,[1]* Robert Jeanjean,[1] Sylvain Lemeille,[2] Michel Havaux,[3] and Cheng-Cai Zhang[1]

Laboratoire de Chimie Bactérienne, IBSM-CNRS, 31 chemin Joseph Aiguier, 13402 Marseille cedex 20, France[1]; Laboratoire Adaptation et Pathogénie des Microorganismes, Université Joseph Fourier, CERMO BP53, 460, rue de la Piscine, F-38041 Grenoble cedex 9, France[2]; and CEA/Cadarache, DEVM, DSV, Laboratoire d'Ecophysiologie de la Photosynthèse, UMR CEA-CNRS 163, Univ. Méditérranée-CEA 1000, F-13108, Saint-Paul-lez-Durance, France[3]

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We establish here that iron deficiency causes oxidative stress in the cyanobacterium *Anabaena* sp. strain PCC 7120. Iron starvation leads to a significant increase in reactive oxygen species, whose effect can be abolished by treatment with the antioxidant tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl). Oxidative stress induced by iron starvation could be a common feature of photosynthetic bacteria.

The availability of iron is crucial for growth, since this element is present in heme and iron sulfur proteins involved in numerous cellular processes, particularly for photosynthetic organisms (3). Many cyanobacteria, such as *Synechococcus elongatus* PCC 7942, *Synechocystis* sp. strain PCC 6803, and *Anabaena* sp. strain PCC 7120, respond to iron limitation by expressing the *isiA* gene (1, 8, 15).

Transcription of *isiA* is also induced by oxidative stress in several cyanobacterial species (6, 9, 14, 16), an observation suggesting that multiple signal input pathways control the expression of this gene (12). Alternatively, iron deficiency could lead to oxidative stress, which then triggers *isiA* expression (10). In this study, we show that iron limitation causes the accumulation of reactive oxygen species (ROS) in *Anabaena* sp. strain PCC 7120 and provides an explanation of the regulation of *isiA* by various factors that cause oxidative damage.

Iron starvation and oxidative stress in *Anabaena* sp. strain PCC 7120 and *Synechocystis*. Expression of *isiA* in *Anabaena* sp. strain PCC 7120 was induced by methyl viologen treatment or by incubation under high light intensities (data not shown). *Anabaena* sp. strain PCC 7120 cultures were iron starved by removing ferric ammonium from BG11 medium and adding the iron chelator 2,2′-dipyridyl at a final concentration of 50 μM. We quantified the amount of ROS with a fluorispectrophotometer (SAFAS) by using the fluorescent probe 2,7-dichlorodihydrodihydrofluorescein diacetate (DCFH-DA; Molecular Probes), which detects hydrogen peroxide, hydroxyl radicals, and peroxynitrite anions. Cells were washed twice with 10 mM phosphate buffer, incubated with the probe at a final concentration of 25 μM, and washed again before the fluorescence was measured (5). The results demonstrated a 10-fold increase in ROS levels in iron-starved cells compared to cells grown with the normal amount of iron. ROS production after methyl viologen treatment served as a positive control of oxidative stress (Fig. 1A). Similar results were obtained with *Synechocystis* sp. strain PCC 6803 under similar conditions (data not shown).

One of the consequences of exposing cells to oxidative damage is the peroxidation of lipids producing light-emitting species, which can be estimated by thermoluminescence (4). The signals obtained with iron-depleted cells were significantly higher than those of iron-sufficient cells (Fig. 1B). Our results indicate that when cyanobacterial cells are iron starved, they also undergo an oxidative stress.

***isiA* transcription in the presence of the antioxidant tempol in *Anabaena* sp. strain PCC 7120.** Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl) has been recently described as an antioxidant molecule (2). Tempol is a low-molecular-weight and membrane-permeable antioxidant that protects many biological systems, such as mammalian cells and bacteria, from oxidative damages. Transcription of *isiA* was analyzed by reverse transcription (RT)-PCR as described previously (15). Expression of the *rnpB* gene was used as a control (Table 1). RNA preparations were treated with DNase, and the efficiency of this treatment was verified by PCR. When iron-depleted cells were grown in the presence of 10 mM tempol, the expression of *isiA* was abolished (Fig. 2A). In contrast, the induction of *alr2119*, whose product is similar to the FeoB protein of *Escherichia coli*, was not influenced by the presence of tempol. Since FeoB is required for iron transport (7), our data suggest that tempol does not change the iron-starved state of the cells (Fig. 2A).

The presence of CP43′ encoded by *isiA* results in a shift of the 680-nm chlorophyll *a* absorption peak (13). Cells starved for iron but incubated with tempol did not exhibit this shift (Fig. 2B). These results are consistent with the suppression of the transcription of *isiA* by tempol in iron-starved cells.

Iron starvation and oxidative stress in heterotrophic bacteria. We measured the ROS levels in *E. coli* cultures grown under normal conditions in MOPS (morpholinepropanesulfonic acid) medium (11) or under conditions of iron deficiency (MOPS medium supplemented with 50 μM of 2,2′-dipyridyl). The ROS production in cells treated with hydrogen peroxide

* Corresponding author. Mailing address: Laboratoire de Chimie Bactérienne, IBSM-CNRS, 31 chemin Joseph Aiguier, 13402 Marseille cedex 20, France. Phone: 00 33 4 91 16 41 88. Fax: 00 33 4 91 71 89 14. E-mail: latifi@ibsm.cnrs-mrs.fr.

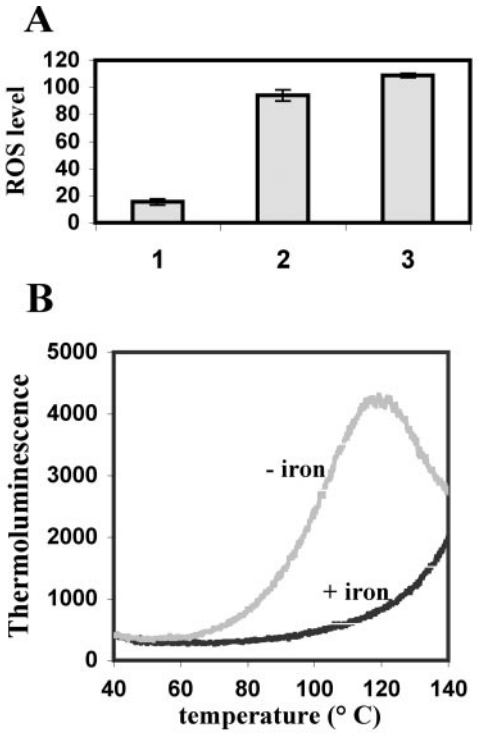

FIG. 1. Oxidative stress under conditions of iron deficiency in *Anabaena* sp. strain PCC 7120. (A) ROS generated by *Anabaena* sp. strain PCC 7120 cells were analyzed after reaction with 2,7-DCFH-DA. The fluorescence intensity was normalized to the optical densities of the samples. Resulting values are presented in arbitrary units. Bars: 1, iron-replete cells; 2, iron-depleted cells; 3, cells treated with methyl viologen. (B) Lipid peroxidation state under oxidative stress induced by iron starvation. Thermoluminescence was measured with cells grown in the presence of iron (black line) or absence of iron (gray line). Experiments were done twice with similar results.

TABLE 1. Sequences of the primers used in this study

Oligonucleotide for indicated gene	Sequence from 5′ to 3′	Target gene
isiA		
Top	GCCCGCTTCGCCAATCTCTC	*isiA* of *Anabaena* sp. strain PCC 7120
Bottom	CCTGAGTTGTTGCGTCGTAT	
rnpB		
Top	AGGGAGAGAGTAGGCGTTGG	*rnpB* of *Anabaena* sp. strain PCC 7120
Bottom	GGTTTACCGAGCCAGTACCTCT	
$feoB_{Ec}$		
Top	AAGATAACTGGCAGGCAACG	*feoB* of *E. coli*
Bottom	ACAGGATAACCGCCAGAATG	
rpoA		
Top	CAGGGTTCTGTGACAGAGTTTC	*rpoA* of *E. coli*
Bottom	CTCGTCAGCGATGCTTGCCGGTGG	
$feoB_{Ana}$		
Top	AATGTGTTCACCAAAACCT	*alr2119* of *Anabaena* sp. strain PCC 7120
Bottom	GTTCACTCCGAATAATATTAA	

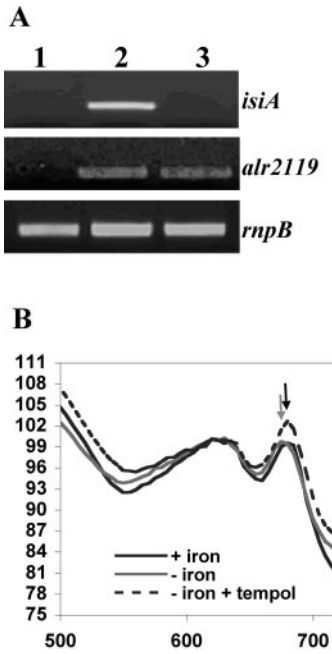

FIG. 2. Effect of tempol on *isiA* expression. (A) RT-PCR analysis of *isiA*, *feoB*, and *rnpB*. Total RNAs were isolated from cells grown in the presence of iron (lane 1), in the absence of iron (lane 2), or in absence of iron and supplemented with 10 mM tempol (lane 3). One microgram of RNA was used in each experiment. Samples were collected at the exponential phase of the PCR. All experiments were repeated twice with similar results obtained. (B) Absorption spectra for cell suspensions with iron (+ iron), without iron (− iron), or without iron but in the presence of tempol (− iron + tempol). The gray arrow indicates a shift of the 680-nm chlorophyll *a* absorption peak.

served as a positive control of oxidative stress. Figure 3A shows that when cells of *E. coli* were iron starved, they did not exhibit a significant increase in the ROS levels. Transcription of the *feoB* gene was analyzed by RT-PCR. The *rpoA* gene, encoding

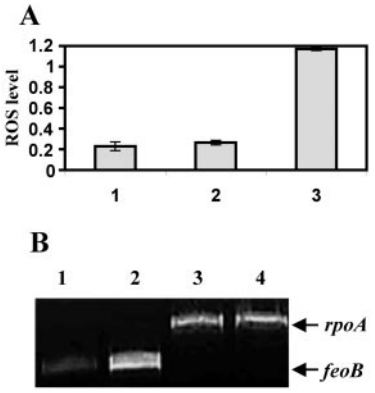

FIG. 3. Oxidative stress under conditions of iron deficiency in *E. coli*. (A) ROS generated by *E. coli* DH5α cells were analyzed by using the fluorescent probe 2,7-DCFH-DA. The fluorescence intensity was normalized to the optical densities of the samples. Resulting values are presented in arbitrary units. Bars: 1, iron-replete cells; 2, iron-depleted cells; 3, cells treated with H_2O_2. (B) RT-PCR analysis of *feoB* and *rpoA* mRNAs. Cells were grown normally (lanes 1 and 3) or under conditions of iron limitation (lanes 2 and 4). One microgram of RNA was used in each experiment. Samples were collected at the exponential phase of the PCR. All RT-PCR experiments were repeated twice, with similar results obtained.

the α subunit of the RNA polymerase, was used as a control (Table 1). The transcription of *feoB* was induced only under conditions of iron deficiency (Fig. 3B), confirming that cells were indeed iron starved under our assay conditions. Similar results were obtained with *Bacillus subtilis* (data not shown).

Our data indicate that iron deficiency creates an oxidative stress which is the major signal inducing *isiA* expression in *Anabaena* sp. strain PCC 7120. Since, under our experimental conditions, oxidative damage induced by iron starvation is not conserved in heterotrophic bacteria, we propose that it could be characteristic of photosynthetic organisms.

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